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In Vivo Dynamics of the Rough Deal Checkpoint Protein during *Drosophila* Mitosis

Renata Basto,[1,2,4] Frédéric Scaerou,[1] Sarah Mische,[3] Edward Wojcik,[3,5] Christophe Lefebvre,[1] Rui Gomes,[2] Thomas Hays,[3] and Roger Karess[1,*]

[1]Centre de National de Récherche Scientifique
Centre de Génétique Moléculaire
Avenue de la Terasse
91198 Gif-sur-Yvette,
France

[2]Departamento de Biologia Vegetal
Centro de Genetica e Biologia Molecular
Faculdade de Ciências da Universidade de Lisboa
1600-Lisboa
Portugal

[3]Department of Genetics, Cell Biology, and Development
University of Minnesota
321 Church Street
Minneapolis, Minnesota 55455

Summary

Rough Deal (Rod) and Zw10 are components of a complex required for the metazoan metaphase checkpoint [1, 2] and for recruitment of dynein/dynactin to the kinetochore [3]. The Rod complex, like most classical metaphase checkpoint components, forms part of the outer domain of unattached kinetochores. Here we analyze the dynamics of a GFP-Rod chimera in living syncytial *Drosophila* embryos. Uniquely among checkpoint proteins, GFP-Rod robustly streams from kinetochores along microtubules, from the time of chromosome attachment until anaphase onset. Prometaphase and metaphase kinetochores continuously recruit new Rod, thus feeding the current. Rod flux from kinetochores appears to require biorientation but not tension because it continues in the presence of taxol. As with Mad2, kinetochore- and spindle-associated Rod rapidly turns over with free cytosolic Rod, both during normal mitosis and after colchicine treatment, with a $t_{1/2}$ of 25–45 s. GFP-Rod coimmunoprecipitates with dynein/dynactin, and in the absence of microtubules both Rod and dynactin accumulate on kinetochores. Nevertheless, Rod and dynein/dynactin behavior are distinguishable. We propose that the Rod complex is a major component of the fibrous corona and that the recruitment of Rod during metaphase is required to replenish kinetochore dynein after checkpoint conditions have been satisfied but before anaphase onset.

*Correspondence: karess@cgm.cnrs-gif.fr

[4]Present address: The Wellcome Trust/Cancer Research United Kingdom Institute of Cancer and Developmental Biology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QR, United Kingdom.

[5]Present address: Virginia Polytechnic Institute and State University, Department of Biology, 213 Engel Hall, Blacksburg, Virginia 24061.

Results and Discussion

The metaphase checkpoint detects improperly attached kinetochores and promotes the inhibition of Cdc20, an activator of the Anaphase Promoting Complex, by the proteins Mad2 and BubR1 [4]. Precisely how the state of the kinetochore is translated into this inhibition of anaphase onset is unclear, but the Rod complex is an essential participant in this process [1, 2, 5].

GFP-Rod Accumulates on Kinetochores and then Streams toward the Poles

Figure 1A (and Movies S1A, S1B, and Figure S1A; available included with this article online) shows the behavior of GFP-Rod during the typical 5 min mitosis of a cycle 12 embryo. During interphase, GFP-Rod is strictly cytoplasmic. After nuclear envelope breakdown (NEB), GFP-Rod accumulates on kinetochores (frame 0:12), and the signal peaks about 40–50 s later (frames 0:48–1:00). A robust current of GFP-Rod along the kinetochore microtubules (KMTs) then develops as it streams toward the poles during the remainder of prometaphase and metaphase (frames 1:00–3:06). During metaphase, kinetochore-associated Rod signal declines slowly from its peak, whereas KMT-associated Rod levels are maintained (Figure S1A). After anaphase onset (frame 3:12), GFP-Rod on KMTs diminishes, and the signal on the migrating kinetochores rises slightly, persists throughout anaphase A, and gradually disappears during anaphase B.

Spindle-associated GFP-Rod is composed of both large particles and finer material, irregularly distributed along the KMTs. Estimated by kymograph analysis (Figure 1B, left), the velocity of fine-particle movement is about 14 $\pm$ 3.5 μm/min (range 10–20, 6 spindles measured). The larger particles (arrow in Figure 1B, right) move at a similar speed. This velocity is consistent with transport mediated by dynein/dynactin [6]. It is significantly faster than the rate of poleward microtubule flux (3–5 μm/min) [7].

Studies of monooriented meiotic chromosomes by immunostaining suggested that redistribution of the Rod complex from kinetochores to KMTs requires bipolar attachment or tension and not simply microtubule capture [5, 8]. GFP-Rod streaming in embryos also begins after bipolar attachment. The kinetochore pair of Figure 1C (frame 01:15, arrow) is monooriented, with both kinetochores brightly fluorescing and no associated streaming. This chromosome suddenly rotates counterclockwise, and the inter-kinetochore distance increases (frame 1:25), indicating it is now bioriented and under tension. Seconds later (frame 1:45), GFP-Rod begins streaming simultaneously from both kinetochores, consistent with a need for bipolar attachment.

Continuous Rod streaming along KMTs throughout metaphase implies continuous recruitment of new GFP-Rod to the kinetochores to feed the current. Indeed, when colchicine is injected into embryos to disrupt mi-

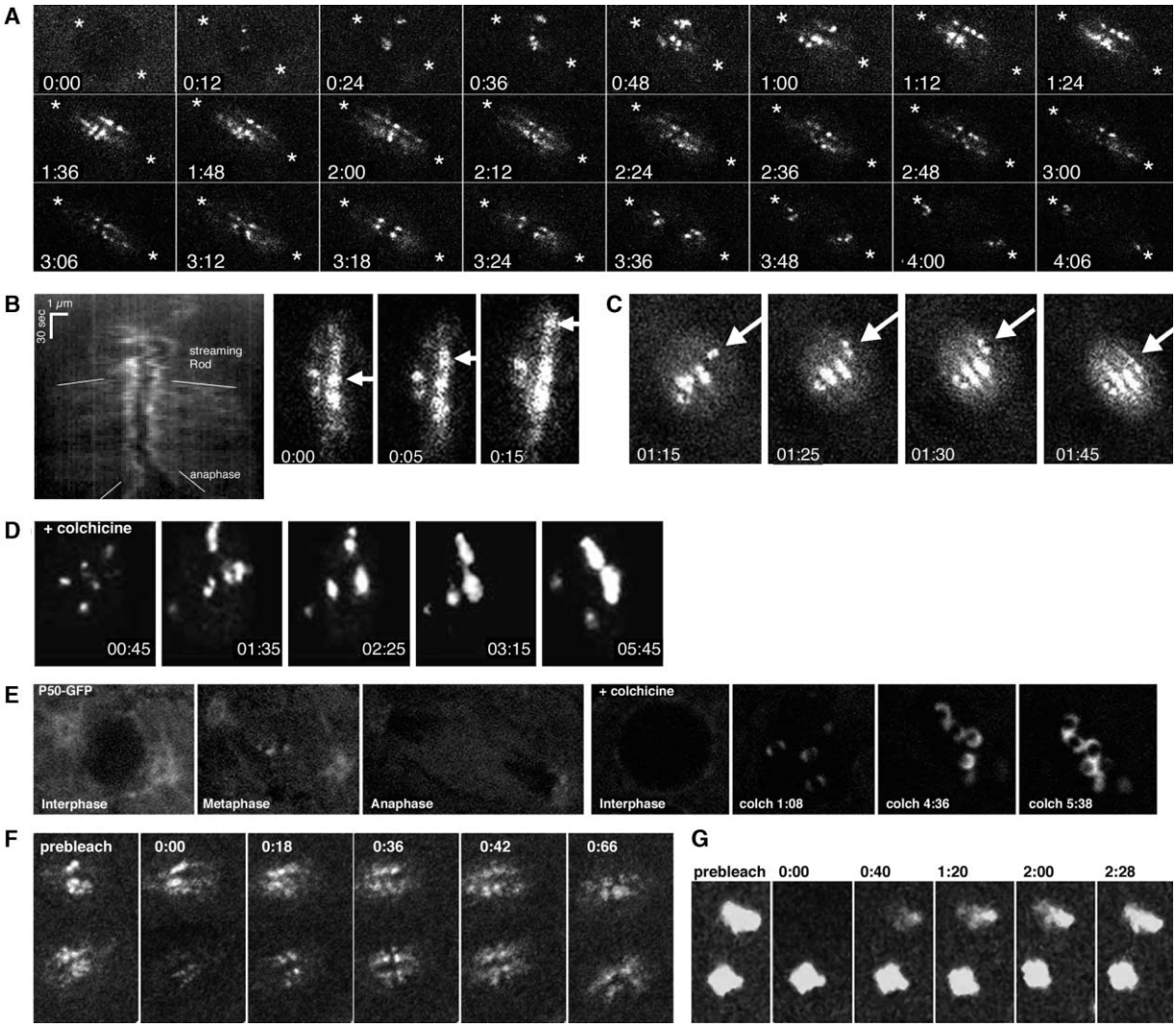

Figure 1. GFP-Rod Associates Dynamically with Kinetochores and KMTs during Mitosis

(A) Rod streams from kinetochores during prometaphase and metaphase. Shown is a syncytial cycle 12 nucleus from a GFP-Rod embryo recorded by time-lapse confocal microscopy (TLCM). Rod is excluded from interphase nuclei (frame 00:00, approximate positions of centrosomes are marked with an asterisk). After NEB (0:12), Rod starts accumulating on kinetochores, with the signal increasing throughout prometaphase until the onset of streaming (0:12–1:12). Once kinetochores have bioriented and aligned, a robust poleward flux of GFP-Rod develops and persists during metaphase (1:00–3:12). At the onset of anaphase (03:12), the signal declines on KMTs but remains strong on kinetochores, (3:18–3:42) before declining during anaphase B. Time shown here and elsewhere is min:s. (See Movies S1A and S1B.)

(B) KMT-associated GFP-Rod is composed of both fine and coarse particles and streams rapidly to the poles. Left: kymograph analysis of GFP-Rod streaming. The lateral streaks extending from the brightly labeled kinetochores correspond to the displacement of GFP-Rod along the KMTs. The average velocity is −14 μm/min. Scale: pixels are 0.07μm by 3 s. Right: A different spindle where a large mass of Rod material (arrow) breaks off the kinetochore, reaching the poles (about 4 μm distance) in 15 s, (−16 μm/min).

(C) The onset of significant streaming is associated with biorientation of kinetochores. In this sequence, an apparently monooriented kinetochore pair (arrow) suddenly rotates, and interkinetochore distance increases; the pair presumably just captured MTs from the opposite pole and bioriented (01:15–01:30). Only then does streaming from this kinetochore pair become evident (01:45).

(D) Colchicine treatment leads to massive accumulation of Rod on kinetochores. GFP-Rod embryos injected with colchicine during interphase of cycle 11. Five minutes after NEB, Rod signal is at least 5–8 times higher than that seen in prometaphase kinetochores of untreated embryos. (See Movie S2 and Figure S1B.)

(E) Dynein/dynactin behavior differs from that of Rod. Left series: in normal interphase (first panel) p50-GFP-labeled dynactin complex associates with centrosomes and astral MTs. During metaphase, kinetochores and KMTs label weakly; anaphase kinetochores also label; see also [7]. Right series: colchicine treatment abolishes the interphase centrosomal and astral signal (first panel); after NEB, p50-GFP accumulates on kinetochores (panels 1:08–5:38), but to a lesser extent than Rod. Time (min:s) marks time from NEB.

(F and G) FRAP analysis reveals rapid exchange of kinetochore bound Rod and free Rod. (F) Within 1 min after a normal spindle has been photobleached, the GFP signal rapidly recovers, first on kinetochores and then on KMTs. In (G) a coalesced kinetochore mass of a colchicine-treated GFP-Rod embryo recovers from photobleaching over 2–3 min. $T_{1/2}$ recovery time is −24 s in untreated spindles and −45 s in colchicine-treated kinetochores (see Figures S2A and S2B).

crotubules, GFP-Rod accumulates greatly on kinetochores (Figure 1D, Figure S1B, and Movie S2), and within 5 min reaches a level at least 5- to 8-fold greater than that on untreated prometaphase kinetochores. GFP-Rod initially forms arcs around such kinetochores, as do other outer-domain components such as dynactin after exposure of PtK cells to nocodazole [9] (Figure 1E, right). However, GFP-Rod tends to coalesce as it accumulates (Movie S2), and within a few minutes individual kinetochores could no longer be resolved.

Comparing Rod Behavior with Dynein/Dynactin

Rod complex is necessary to recruit cytoplasmic dynein/dynactin to kinetochores [3], and dynein is required for the KMT transport of Rod complex and other outer domain proteins [6, 10]. However, Rod complex and dynein/dynactin behavior are not identical (Figure 1E). A GFP-tagged version of the dynactin subunit p50 associates with *Drosophila* embryonic centrosomes during interphase, and during prometaphase it binds kinetochores and migrates along KMTs [6]. Immunostaining shows that dynein/dynactin, unlike Rod, diminishes rapidly upon microtubule capture [9, 11]. Moreover, in vivo, GFP-dynein/dynactin is substantially weaker on kinetochores and KMTs during embryonic metaphase than is GFP-Rod [6] (compare Figures 1A and 1E). Finally, although dynein/dynactin accumulates significantly on colchicine-treated kinetochores (Figure 1E, right series), it never forms the enormous mass of material seen with GFP-Rod (Figure 1D). Together, these results suggest that kinetochore Rod complex and dynein/dynactin can act partially independently of one another.

Kinetochore Bound Rod Rapidly Exchanges with Free Rod

Both Mad2 and Cdc20 transit rapidly through kinetochores with a half-life (time of residence) on kinetochores of 24–28 s [12] and 5–10 s [13, 14], respectively. These rapid turnover times are independent of microtubules and have been used to support the model that Mad2 and Cdc20 are involved in disseminating information about the kinetochore linkages (the "wait anaphase" signal) [12, 14, 15].

To determine if kinetochore bound GFP-Rod is also rapidly exchanging with cytosolic Rod, we performed FRAP (fluorescent recovery after photobleaching) analysis of normal and colchicine-treated mitotic embryos (Figures 1F and 1G). Bleached prometaphase spindles (Figures 1F and S2A) rapidly regain their original Rod fluorescence within 60 s, with the signal recovering first on the kinetochores and later on the spindle fibers. Analysis of photorecovery kinetics (Figure S2A) gives a half-life time-of-residence ($t_{1/2}$) of Rod on the mitotic apparatus as 24 $\pm$ 3 s; (four determinations).

Although Rod's simultaneous recruitment to and transport from the kinetochores along the KMTs might explain such a short transit time, we found that Rod turned over rapidly even in the absence of microtubules. A photobleached kinetochore mass of a colchicine-arrested mitotic figure recovers in about 2.5 min (time-of-residence $t_{1/2}$ = 45 $\pm$ 7s; 6 determinations, Figures 1G and S2B) typically plateauing at 60%–100% of the original signal.

Thus, FRAP analysis reveals that kinetochore bound Rod is constantly exchanging with free Rod in the cytosol. In normal metaphase spindles, the kinetochore bound Rod population leaves by two routes: via a dynein- and KMT-mediated transport and via a microtubule-independent pathway that releases Rod directly into the cytosol, as is the case with Mad2 and Cdc20. The 24 s half-life for Rod on normal spindles probably therefore reflects the activity of both pathways, each contributing about equally to the turnover rate (see Supplemental Results and Discussion).

Taxol Treatment Does Not Stop Rod Streaming

Suppression of microtubule dynamics relaxes the tension exerted by KMTs on bioriented chromosomes [16], which may activate the metaphase checkpoint. Taxol-treated vertebrate cells arrest in metaphase with normal-looking spindles and chromosomes aligned in a compact metaphase plate [17]. To assess the impact of microtubule dynamics on the checkpoint and on Rod behavior, we injected embryos with 5 mM taxol and monitored microtubules, chromosomes, cyclin B degradation, and Rod by using fluorescently tagged proteins (Figure 2 and Movies S3A–C).

Depending on its concentration, taxol produced two very different effects on syncytial nuclei as they entered mitosis. High doses near the site of injection (the "proximal" half of the embryos in Figure 2A) produced a mitotic arrest similar to that seen with colchicine; Rod accumulated on kinetochores, chromosomes remained condensed, and cyclin B was stable for at least 30 min (the end of observation). This mitotic arrest was caused by a block in spindle assembly; MTs were largely excluded from the nuclear regions (Figure S3 and Supplemental Results and Discussion), and consequently, few MTs were available for kinetochore capture.

In the injection-distal half of the same embryos, normal-looking bipolar spindles formed (Figure 2A and Movies S3A and S3B). These spindles did exit mitosis, degrade cyclin B, and reform nuclei, but only after a significant delay (4–8 min after NEB, rather than 2–3 min in untreated embryos). Thus, although taxol can induce prolonged mitotic arrest in fly embryos, it does so by blocking spindle assembly. Bipolar spindles themselves are delayed in mitosis by taxol but are not arrested. (See Supplemental Results-Discussion.)

GFP-Rod continues to stream along KMTs of taxol-treated bipolar spindles (Figure 2B and Movie S3C). However, about 3 min after NEB, new Rod recruitment to the kinetochores ceases (frame 2:15 and thereafter). The remaining Rod migrates along the KMTs and accumulates abnormally at the poles, where it eventually disappears. The delayed mitotic exit (monitored by cyclin B degradation in separate embryos) corresponds roughly to the time when Rod is disappearing from the mitotic apparatus.

That Rod streaming continues in taxol argues that streaming does not require microtubule dynamics or tension. Yet observations of monooriented chromosomes in untreated embryos (Figure 1) and the immuno-

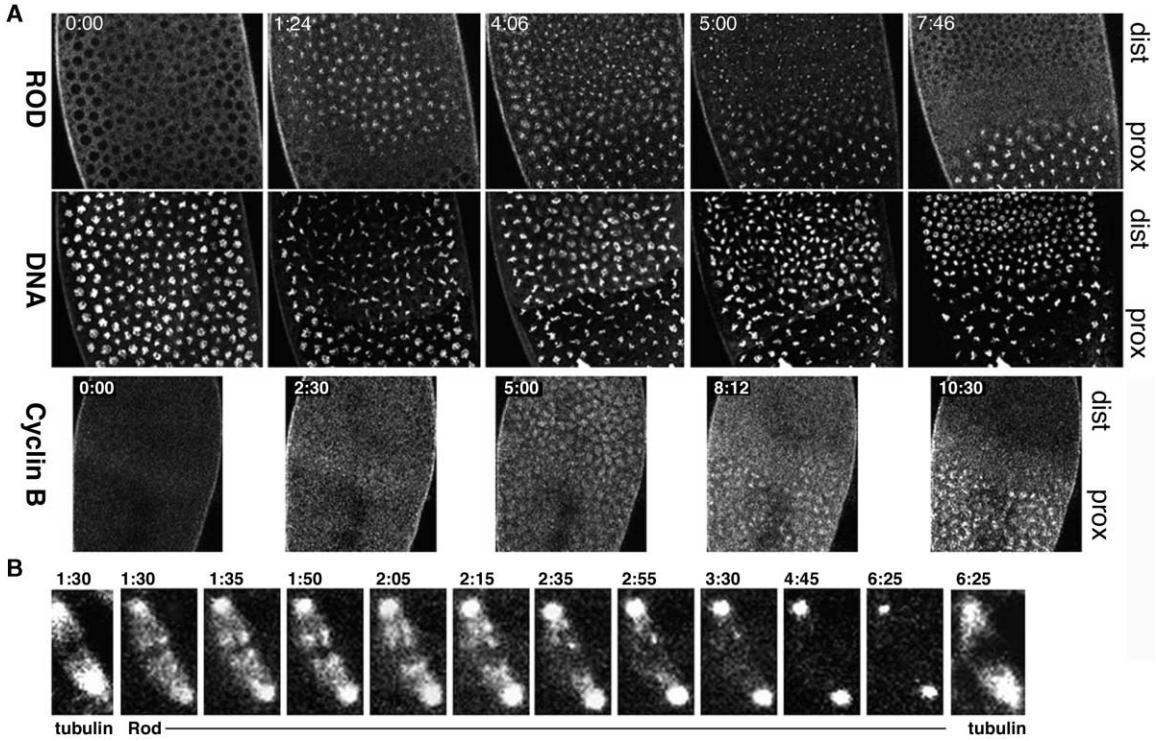

Figure 2. Taxol Produces Concentration-Dependent Effects on the Mitotic Cycle and Alters Rod Behavior on Spindles

(A) The central region of a cycle 12 embryo doubly labeled with GFP-Rod (top row) and rhodamine histone (second row), injected posteriorly (bottom of frames) with 5 μM taxol. Distal to the site of injection, chromosomes condense and Rod enters the nuclei, where it associates with the mitotic apparatus (1:24). The distal region exits mitosis but with a significant delay relative to untreated embryos (2–6 min, depending on the experiment, frames 5:00 and 7:46). The reforming nuclei appear as black holes where GFP-Rod is excluded. The injection-proximal region enters mitosis with a slight delay. Nuclei condense and Rod accumulates, but no spindle develops (Figure S3), and the nuclei are arrested in a mitotic state. (Third row) GFP-cyclin-B-expressing embryo [22] injected with taxol. The distal half of the embryo begins mitosis, with cyclin B accumulating around the spindles (2:30–5:00). The distal nuclei exit mitosis (cyclin B signal disappears, frames 8:12–10:30), whereas proximal nuclei remain in a mitotic state with elevated cyclin B.

(B) A sequence from the injection-distal region of a GFP-Rod embryo (labeled with rhodamine-tubulin). Rod streaming along KMTs continues but accumulates abnormally at the poles (1:30–2:15). At about this time, new Rod recruitment to the kinetochore ceases, and the remaining GFP-Rod on the spindle declines. The first and last frames are tubulin images of the same spindle from the beginning and end of the sequence, showing that despite the evolution of the Rod image, the spindle retains a metaphase form. See also the Supplemental Data.

staining studies of univalent meiotic chromosomes [5, 8] suggest that Rod flux only begins after biorientation, which is usually considered synonymous with the establishment of bipolar tension. We have confirmed (by immunostaining) that on monopolar spindles in several mitotic *Drosophila* mutants, including *polo, mgr, aurora,* and *asp*, Rod accumulates on both attached and unattached kinetochores and is nearly always absent from the KMTs (our unpublished data). It is not obvious how kinetochores might detect when they are bioriented in the absence of tension. One model would be that Rod streaming is promoted by a strict perpendicular linkage between kinetochores and microtubules, a geometry most easily achieved by biorientation.

The effects of taxol on Rod behavior are similar to those of energy depletion on Mad2 in PtK cells [10, 12], which also blocks new recruitment to kinetochores but not transport along KMTs and promotes an abnormal accumulation of the protein at the poles. Because both taxol and energy depletion suppress MT dynamics, it seems likely that the block to new Rod recruitment and retarded release of Rod from the poles are both consequences of stabilizing spindle MTs. These results suggest (1) that Rod kinetochore recruitment rates may normally vary as a function of the changes in MT-kinetochore linkages that occur during attachment and congression of chromosomes on the spindle; and (2) that MT disassembly at the poles facilitates release of Rod from the spindle.

Dynein/Dynactin Physically Associates with the Rod Complex In Vivo

The p50 subunit of dynactin interacts with Zw10 in a two-hybrid assay [3]. However, the 700 kD Rod complex [6] apparently does not contain components of the dynein-dynactin complex [6], and there is no evidence for a physical interaction in vivo between dynein/dynactin and the Rod complex. To test for such an interaction, we performed coimmunoprecipitations with GFP-Rod and p50-GFP embryo extracts (Figure 3). Rod was detectably coimmunoprecipitated with p50-GFP protein, along with dynactin component p150-Glued and dynein heavy chain (DHC). Similarly, anti-GFP coprecipitated a fraction of the p150-Glued and DHC in immunoprecipi-

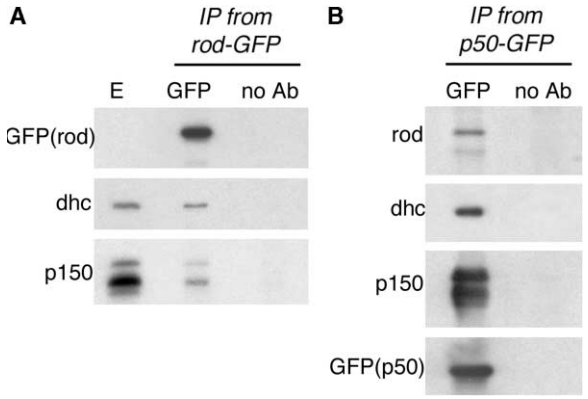

Figure 3. Rod Associates with Dynactin In Vivo

Western blots of anti-GFP immunoprecipitates and control (no antibody) precipitates from GFP-Rod embryos and p50-GFP embryos. (A) DHC and p150-Glued coprecipitate with GFP-Rod. (p50 cannot be detected here because it comigrates with IgG heavy chains.) DHC and p150 are present in free pools and are not quantitatively precipitated with GFP-Rod. The concentration of GFP-Rod is greatly enriched in the IP pellet, relative to total extract (lane E).

(B) Reciprocally, Rod, p150, and DHC coprecipitate with the GFP-tagged p50 protein. Antibodies probing the blots are listed to the left of panels.

tates of GFP-Rod embryos. In neither case was the coprecipitation quantitative, indicating that only a fraction of the Rod in a cell is associated with dynactin at a given moment. This is also consistent with the fact that the mitotic behaviors of p50-GFP and GFP-Rod are similar but not identical.

Conclusions

The Rod complex possesses several of the dynamic features that in Mad2 are believed to be related to its function in generating and disseminating the "wait anaphase" inhibitory signal: accumulation on unattached kinetochores; relative depletion from kinetochores after chromosome alignment; migration along KMTs toward the poles; and rapid exchange between kinetochore bound and unbound material, even in the absence of microtubules. However, Rod also differs profoundly from Mad2. After chromosome alignment when checkpoint conditions are satisfied, Mad2 becomes essentially undetectable on aligned kinetochores [2, 12] whereas Rod is continuously recruited to kinetochores and shunted off along the KMTs until anaphase onset.

The taxol experiments revealed that rates of Rod recruitment to the kinetochores and release from the spindle poles are sensitive to changes in microtubule dynamics. The balance among recruitment, transport, and release presumably explains the accumulation of Rod on unattached kinetochores in prometaphase or after colchicine treatment as well as its relative depletion from kinetochores after capture and alignment on the spindle; when the combined dispersion rates are greater than the recruitment rate, Rod no longer accumulates. These dynamics are summarized in Figure 4.

Without MTs, kinetochores accumulate Rod complex and dynein/dynactin to levels far exceeding those seen in normal prometaphase, whereas outer-domain checkpoint proteins such as Mad2, BubR1, and CenpE accumulate only to prometaphase levels. This suggests that although the maximum number of binding sites per kinetochore for these proteins may be fixed, the Rod complex and its associated proteins may self-assemble and oligomerize on the kinetochore, thus allowing for greater expansion of the kinetochore outer domain. The apparent coalescence of GFP-Rod material around kinetochores (Figure 1D and Movie S2) supports this idea.

The robustness of the Rod flux during metaphase is unique among checkpoint proteins. Why is Rod still recruited to aligned kinetochores until the moment of anaphase onset? Perhaps it is related to the role of the Rod complex in recruiting dynein/dynactin to kinetochores.

Dynein is implicated in the KMT transport of many outer kinetochore proteins, including Mad2, BubR1, and

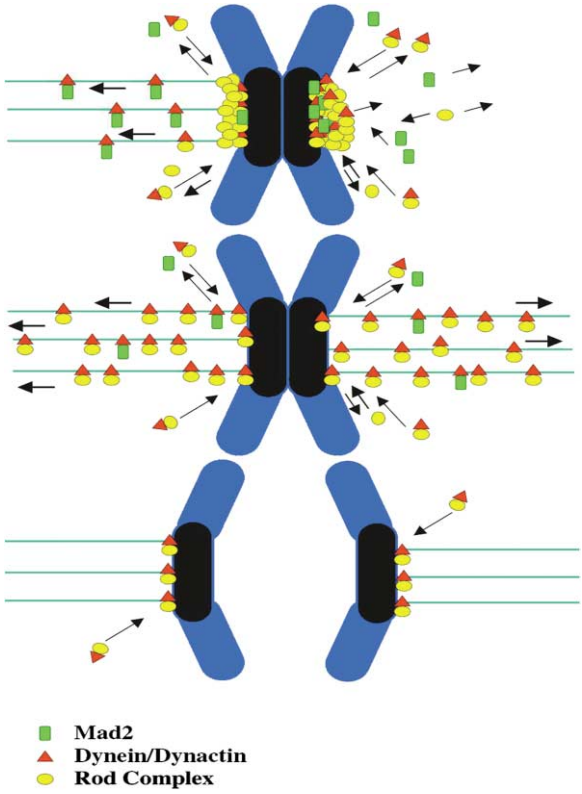

Figure 4. Summary of Rod Behavior during Mitosis

Rod/Zw10 complex (along with dynein/dynactin complex) and Mad2 are recruited to kinetochores but can leave by either of two routes: direct dissociation from the kinetochore or dynein/dynactin-mediated transport along KMTs. The relative rates of recruitment, dissociation, and transport determine their abundance at the kinetochores. During prometaphase, the Rod complex, dynein/dynactin, and Mad2 all accumulate on unattached kinetochores (top). Microtubule capture leads to Mad2 depletion [10–12] as it is carried off by dynein/dynactin. Rod complex accumulation continues, replenishing kinetochore dynein. In bioriented chromosomes (middle), a flux of the Rod complex material develops, as new kinetochore recruitment is balanced by dynein/dynactin-mediated poleward transport. The continuing recruitment of the Rod complex during metaphase may serve to maintain adequate dynein/dynactin complex on kinetochores for assisting chromatid movement during anaphase (bottom).

CenpE [10], as well as the Rod complex [6], and this transport may be one way that the checkpoint mechanism is shut off [6, 10]. But dynein also appears to be required for efficient anaphase chromatid migration in *Drosophila* [18, 19]. Paradoxically, far less dynein is detectable at kinetochores during anaphase than during prometaphase [6, 9]. We suggest that kinetochore dynein is constantly replenished via the Rod complex [3] during metaphase to ensure that enough will be available for proper chromatid migration at anaphase (Figure 4). The dynein/dynactin may, however, be only transiently available to take its chromatid cargo. If the chromatid is not ready (prior to anaphase onset), the dynein leaves without it, taking other outer-domain proteins along. A continuous ski lift provides a useful analogy; seats are always available at the bottom of a mountain to tow a skier, but the seats leave empty if nobody is waiting.

Finally, we note that among the outer-domain proteins, the Rod complex comes closest to displaying the behavior expected for a component of the fibrous corona, a structure defined principally by electron microscopy [20, 21]. Only the Rod complex shows the corona-like accumulation (possibly oligomerization) on MT-free kinetochores and the robust signal extending along KMTs during metaphase. Thus, the fibrous corona may not be identical to the outer domain but may be primarily composed of Rod, Zw10, dynactin, and their associated proteins, and rather than stretching along KMTs, it may be recruited to the kinetochore and migrate toward the pole.

Supplemental Data

Supplemental Data including Experimental Procedures, movies (S1A, S1B, S2, S3A-S3C), figures (S1A, S1B, S2A, S2B, S3), quantitation of GFP-Rod, and additional Results and Discussion are available at http://www.current-biology.com/cgi/content/full/14/1/56/DC1/.

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